



PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Rogalska et al.

Serial No.: 10/764,270

Filed: January 23, 2004

For: METHOD OF BINDING A
COMPOUND TO A SENSOR SURFACE

Confirmation No.: 6555

Examiner: A. Noguerola

Group Art Unit: 1795

Attorney Docket No.: 2183-6294US

NOTICE OF EXPRESS MAILING

Express Mail Mailing Label Number: EV962545121US

Date of Deposit with USPS: May 23, 2008

Person making Deposit: Robert J. Gueck

**TRANSMITTAL OF PRIORITY DOCUMENT AND
NOTIFICATION OF SMALL ENTITY STATUS**

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

On the Notice of Allowability of February 28, 2008, the examiner indicated that the priority document had not been received. A copy of EP01202802 is enclosed.

This application qualifies for small entity status.

Respectfully submitted,

Allen C. Turner
Registration No. 33,041
Attorney for Applicants
TRASKBRITT, P.C.
P.O. Box 2550
Salt Lake City, Utah 84110-2550
Telephone: 801-532-1922

Date: May 23, 2008
ACT/bv

**Bescheinigung**

Die angehefteten Unterlagen stimmen mit der ursprünglich eingereichten Fassung der auf dem nächsten Blatt bezeichneten europäischen Patentanmeldung überein.

Certificate

The attached documents are exact copies of the European patent application described on the following page, as originally filed.

Attestation

Les documents fixés à cette attestation sont conformes à la version initialement déposée de la demande de brevet européen spécifiée à la page suivante.

Patentanmeldung Nr.**Patent application No.****Demande de brevet n°**

01202802.3 / EP01202802

The organization code and number of your priority application, to be used for filing abroad under the Paris Convention, is EP01202802.

Der Präsident des Europäischen Patentamts;
Im Auftrag

For the President of the European Patent Office
Le President de l'Office européen des brevets
p.o.

R.C. van Dijk

Anmeldung Nr.: **01202802.3**
Application no.: **01202802.3**
Demande no.:

Anmeldetag: **23.07.01**
Date of filing: **23.07.01**
Date de dépôt:

Anmelder / Applicant(s) / Demandeur(s):

Applied NanoSystems B.V.
P.O. Box 100
9700 BC Groningen/NL

Bezeichnung der Erfindung / Title of the invention / Titre de l'invention:
(Falls die Bezeichnung der Erfindung nicht angegeben ist, siehe Beschreibung.
If no title is shown please refer to the description.
Si aucun titre n'est indiqué se référer à la description.)

Method of binding a compound to a sensor surface using hydrophobin

In Anspruch genommene Priorität(en) / Priority(Priorities) claimed / Priorité(s) revendiquée(s)
Staat/Tag/Aktenzeichen / State/Date/File no. / Pays/Date/Numéro de dépôt:

Internationale Patentklassifikation / International Patent Classification / Classification internationale de brevets:

G01N33/00

Am Anmeldetag benannte Vertragstaaten / Contracting States designated at date of filing / Etats contractants désignées lors du dépôt:
AT BE CH CY DE DK ES FI FR GB GR IE IT LI LU MC NL PT SE TR

EP 1033-A1/ho

Method of binding a compound to a sensor surface using hydrophobin

The present invention relates to a method of binding a compound to a sensor surface, said method comprising the step of adsorbing hydrophobin to said sensor surface.

Generally, there is a need in the art to bind compounds to a sensor surface. To achieve this, it is known to adsorb hydrophobin to the surface of a sensor, and to chemically link a compound to the adsorbed hydrophobin. In particular, Wessels et al. (Advances in Microbial Physiology, 38, pp. 1-45 (1997)) suggest attaching small ligands to a layer of hydrophobin (p. 35). The (only) example given relates to coupling a protein molecule onto a layer of hydrophobin present on a gold surface.

Coating the surface with a hydrophobin may reduce the sensitivity of the sensor, as less surface area is available, or reactions to be detected take place at a greater distance from the sensor surface.

The object of the present invention is to provide a method according to the preamble with improved sensitivity.

To this end, the method according to the present invention is characterized in that the compound is chosen from the group consisting of i) an electroactive compound, an ii) a compound capable of being converted into an electroactive compound, said method comprising the steps of
a) coating the electrode with a hydrophobin, and
b) contacting the compound with the hydrophobin
to form a hydrophobin coating containing said compound in a non-covalently bound form.

A particular type of sensor is the electrochemical sensor, comprising an electrode as the sensor surface. Coating an electrode with a hydrophobin would result in reduced access of electroactive compounds to the surface of said electrode. Surprisingly, we have found that it is possible to non-covalently incorporate a compound in said hydrophobin coating. The compound remains for a substantial time in the hydrophobin coating, as evidenced by experiment. The electro-

active nature of the compound improves the sensitivity of the electrode in comparison with a hydrophobin coating not containing said compound.

In the present invention, the term "hydrophobin" refers to a protein capable of coating a surface, rendering a hydrophobic surface hydrophilic, and often vice versa, and having a length of up to 125 amino acids, consisting of a member from the group chosen from i) a protein having a conserved sequence

10 $X_n-C-X_{5-9}-C-C-X_{11-19}-C-X_{8-23}-C-X_{5-9}-C-C-X_{6-18}-C-X_m$
 wherein X, of course, represents any amino acid, and n and m, of course, independently represent an integer as disclosed by Wessels et al. (ref. 8). These hydrophobins are typically isolated from fungi like Schizophyllum commune (ref. 8); and
 15 ii) a protein comprising a polypeptide having at least 40% identity and at least 5% similarity to at least one polypeptide chosen from the group consisting of i) amino acids 29 - 131 of SEQ NO. 1 and ii) amino acids 29 - 133 of SEQ. NO. 2. Such a protein may be derived from a filamentous bacterium,
 20 in particular a bacterium capable of forming aerial hyphae such as an Actinomycete, and more specifically the filamentous bacterium may be a Streptomyces species. A Streptomyces species from which the protein may be isolated using standard procedures for the isolation of hydrophobins, is a Streptomyces species which has been transformed with a construct that can be isolated from an E. coli strain which has been deposited on 14 March, 2000 under accession number CBS 102638 with the Centraalbureau voor Schimmelcultures (Oosterstraat 1, P.O. Box 273, 3740 AG Baarn, the Netherlands). This is disclosed in PCT/NL01/00268.

In the above definition, the term "identity" used in association with polypeptide is defined, in accordance with the state of the art, as having exactly matched amino acid residues. Here, sequences may comprise insertions or deletions. The term "similarity" used in association with polypeptide denotes conservative substitutions. Conservative substitutions are substitutions in which one amino acid is replaced with another, where the following amino acids are con-

sidered similar:

A, S, T;
D, E;
N, Q;
5 R, K;
I, L, M, V;
F, Y, W.

The term "electro-active" is defined as a compound which can undergo changes in the oxidation state, i.e. undergo a redox reaction. An electro-active compound will have a lower molecular weight than the hydrophobin, and more in particular it will have a MW of less than 2000, and more preferably less than 1000. Compounds capable of being converted into an electroactive compound are known in the art. 10 They may become electroactive after, for example, irradiation with light. This allows the electroactive compound to be made available at a desired time. Some of the advantages of the use of such a compound are 15

- more accurate measurements (background measurements can be 20 made before the electroactive compound is released)
- reduced consumption of electroactive compound. Generally, the compound capable of being converted into an electroactive compound will have a molecular weight as specified above.

In particular, the compound is a hydrophobic compound or a compound containing a hydrophobic anchor. 25

Such compound are among the compounds most stably maintained in the hydrophobin coating. It is thought that a planar hydrophobic compound or anchor may be beneficial. In the present application the term "anchor" is understood to 30 mean a part of the compound, said part having a side and/or moiety lacking hydrophilic groups. It is also thought that the absence or a reduced number of negative and/or positive charges is advantageous. If charge is present, it is preferably from weakly acidic or basic groups, which can release or 35 accept a hydrogenium ion to eliminate the charge.

Advantageously, a second compound is bound covariantly to the hydrophobin, the second compound being an electroactive compound.

According to an alternative embodiment, second compound is bound non-covalently to the hydrophobin, through a third compound being an intermediate compound having affinity for the second compound, said second compound being an electroactive compound.

Both these methods allow for a (more) selective measurement. With the ("first") compound being present, good sensitivity is achieved even though the second compound is at a distance from the electrode surface.

Hence, preferably the second compound is a redox enzyme or a light receptor.

A light receptor may, for example, be a haem-group or a chlorophyl-group.

The invention also relates to an electrode coated with a hydrophobin, the hydrophobin containing a compound being an electroactive compound.

The electrode will be manufactured using any embodiment of the method according to the invention.

The electrode may be, for example a gold or platinum electrode, and in particular, the electrode is a glassy-carbon electrode (GCE), a glass electrode (GE) or a Thin Mercury Film Electrode (TMFE).

The invention also relates to a method of performing a measurement with an electrode coated with hydrophobin, characterized in that an electrode according to the invention is used.

Finally, the invention relates to a sensor comprising an electrode according to the invention.

Such a sensor may comprise further means for conducting a measurement, such as lead wires, integrated or non-integrated devices such as an amplifier, a reference electrode, signal processing means, as is well known in the art.

The invention will now be illustrated by way of example and with reference to the drawing in which

Fig. 1a-c show cyclic voltammograms showing the difference between bare (1) and hydrophobin-modified (2) electrodes (GE, GCE, TMFE respectively);

Fig. 2a,b show cyclic voltammograms using ferrocya-

nate ions as a probe to check the blocking properties of hydrophobin;

Fig. 3a,b are similar to fig. 1 and show the effect of adsorption of ubiquinone Q10 adsorbed to a GC-electrode (GCE);

Fig. 4 shows the effect of diazobenzene on a cyclic voltammogram for a GCE; and

Fig. 5 is similar to fig. 4, except that the effect of ubiquinone Q0 is shown.

10

Experimental Section

Production and purification of the hydrophobin. HYDPt-1 hydrophobin was produced in *Escherichia coli* as a recombinant polypeptide of 13.7 kDa (ref. 1). Briefly, the *hydPt-1* cDNA was cloned in the pQE30 plasmid (Qiagen, Germany) to produce a fusion protein with a His-tag motif. Extraction was performed as described previously (ref. 1). After chromatographic purification on a Ni^{2+} affinity column, the HYDPt-1 polypeptide was concentrated in 10 mM Tris-HCl by ultrafiltration.

Chemicals and Solutions. All chemicals were of analytical grade. Tris(hydroxymethyl)aminomethane (Tris), dimethylol-formamide (DMF) and LiOH were from Fluka. Coenzymes Q0 (2,3-dimethoxy-5-methyl-1,4-benzoquinone) and Q10 (ubiquinone 50) were from Sigma. Diazobenzene was from Reachim, Hungary. Methanol, citric acid and $\text{K}_3[\text{Fe}(\text{CN})_6]$ were from POCh, Poland. All solutions were prepared daily. Distilled water was passed through a Milli-Q water purification system. The surface tension was 72.5 mN m^{-1} at 20°C and final resistivity was $18.3 \text{ M}\Omega \text{ cm}^{-1}$.

Electrochemical experiments. Voltammetry experiments were done at 20°C in three - electrode arrangement, with a calomel reference electrode, platinum foil counter electrode and GE, GCE or TMFE as working electrodes. Eco Chemie AUTOLAB PGSTAT 30 system was used as the potentiostat with an IBM PC and Eco Chemie software. In order to prepare the TMFE, a silver wire or silver disk electrode, precleaned in concentrated

perchloric acid, was touched to a drop of mercury and cathodically polarized in 0.1 M KOH to obtain a shining and uniform layer of mercury, ca. 1 mm thick. The GE and GCE were polished on Buehler polishing papers and paste, and the GE 5 was then cleaned in concentrated nitric acid. The electrodes were coated with the self-assembled hydrophobin upon contact with the surface of solutions containing 2 μ g of hydrophobin per 1 ml of 10 mM Tris-HCl buffer. The time of the hydrophobin self-assembly and the conditions of Q0, Q10 and diazoben-10 zene adsorption on hydrophobin coated electrodes are given below.

Barrier properties of HYDPt-1 films on gold and glassy carbon electrodes. The properties of HYDPt-1 layers adsorbed on hydrophilic and hydrophobic solid surfaces were compared 15 using three different electrode substrates, namely GE, GCE and TMFE. The hydrophilic GE surface was modified with HYDPt-1 by self-assembling the protein at the liquid-air interface, followed by adsorption of the layer to the gold surface. The protein was assembled from 10 mM Tris-HCl buffer, pH 7.0, 20 containing 2 μ g/ml hydrophobin, and adsorption to the surface was achieved by lifting the electrode up through the interface, or by a horizontal touching of the hydrophobin-covered water surface with the electrode. Figure 1 shows the cyclic voltammograms recorded using the bare (1) and hydrophobin 25 modified (2) electrodes. Figure 1a allows the comparison of the bare (1) and covered (2) GE. The curves are similar in that no decrease of background current is observed, and no peaks appear in the voltammogram. The presence of the hydrophobin layer on the electrode surface is evidenced by the inhibition of the final increase of anodic current due to gold 30 oxidation. HYDPt-1 is inert in a wide range of potentials and does not lead to a decrease of capacity currents which means that the protein layers formed on the electrode are not as dense and highly blocking, as the layers of, e.g., alkane-35 thiols (ref. 2). The extent of blocking is not changed even after 24 hours of self-assembly.

Wessels and Wösten observed that the SC3 hydrophobin had much higher affinity to hydrophobic than to hydrophilic sur-

faces (ref. 3, 4). Two types of electrodes, GCE and TMFE, were therefore chosen as model hydrophobic surfaces to check the behavior of HYDPt-1. The results of self-assembly are shown in Figures 1b and 1c respectively. In both cases the 5 background currents become much smaller after modification (2), demonstrating that the coverage of GCE and TMFE with HYDPt-1 is much higher, compared to that of the gold substrate. The protein layers are stable and firmly attached to the electrode substrate, as indicated by the GCE voltammogram 10 which does not change over several weeks. In the case of TMFE, high quality films are formed even when the time of self-assembly is decreased from 24 hours to 20 minutes (fig. 1c). The capacity of the modified TMFE is significantly lowered, and the onset of the mercury oxidation current is 15 shifted towards more positive potentials, revealing strong blocking properties of the hydrophobic layer. An additional peak appears in the TMFE voltammogram at -0.58V. This peak corresponds to the reduction of mercury cysteinate formed on the electrode surface upon oxidation of mercury in the presence of cysteine thiol groups present in the protein.

20 **Stability of HYDPt-1 layers in solutions of different pH.** The dependence of stability and blocking properties of HYDPt-1 layers on the pH of the solution was checked by recording multiple cyclic voltammograms using all electrodes in 25 solutions of pH 2.2 (citric acid), pH 4.7 (citric acid / LiOH), pH 7.0, pH 10.2 (Tris), and pH 12.1 (LiOH). The HYDPt-1 layer remained well attached to the electrode surfaces in all solutions studied, and the blocking effect on various substrates followed the behavior observed at pH 7.0.

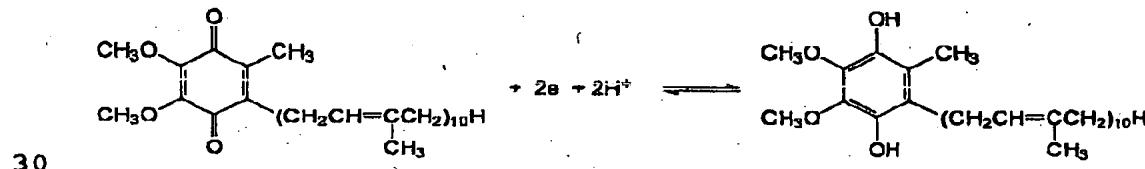
30 **Probing blocking properties of HYDPt-1 layers using ferrocyanate as the electrochemical probe.** The ability of small hydrophilic anions to access the electrode surface through the HYDPt-1 layer was checked using ferrocyanate ions as the electrochemical probe. Cyclic voltammograms were recorded in 35 0.1 M / HClO₄ solution containing 0.75 mM K₃Fe(CN)₆ (Figure 2). The voltammograms recorded for K₃Fe(CN)₆ using HYDPt-1 coated (2) electrodes are different, compared to those obtained with a bare (1) GE (fig. 2a) or GCE (fig. 2b). The

currents are much lower and the voltammetric curves are more sigmoidal in shape. This behavior establishes that the extent of coverage of both electrodes by HYDPt-1 is high, and that the probe has a limited access to the electrode surface. The 5 transition from peaked to sigmoidal shape is expected when the access sites are dispersed (ref. 5, 6), and when spherical diffusion becomes the major process for transporting the molecules to the electrode surface, as distinct from the linear diffusion observed for large bare electrodes.

10 The GCE surface is blocked more efficiently than the gold surface, as shown with the experiments performed in pure supporting electrolyte solution. The latter results confirm a higher affinity of HYDPt-1 towards hydrophobic surfaces.

Hydrophobin as a "molecular glue" for immobilizing molecules on the electrode surface. Wösten and de Vocht suggested that hydrophobin might be used to attach cells to hydrophobic surfaces in medical and sensing devices (ref. 7). In the present work we checked the ability of HYDPt-1 to bind through sorption different types of electroactive molecules to electrode surfaces. The long hydrocarbon chain ubiquinone (Q10) was used as a model hydrophobic molecule. Firstly, the HYDPt-1 layer was self-assembled on the GCE from the usual solution (2 µg/ 1 ml Tris buffer, pH 7.0). Next, self-assembly of Q10 was carried out from a solution containing 1 mg of Q10 in 1 25 ml DMF.

Electroreduction of ubiquinone Q 10 immobilized on electrodes modified with HYDPt-1. In neutral aqueous solution ubiquinone undergoes reduction, according to the following scheme:

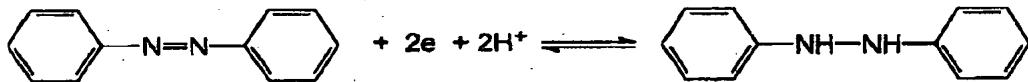


Scheme 1

The voltammetric curve obtained with ubiquinone Q10 adsorbed

on the HYDPt-1 modified GE (2 in fig. 3a) and GCE (2 in fig. 3b) is shown. In fig. 3, 1 denotes an electrode covered with hydrophobin only. The shape of the curve and the linear dependence of the peak currents on the scan rate points to surface immobilization of ubiquinone. The GCE substrate covered with HYDPt-1 was found to bind Q10 in a very stable way, giving rise to ubiquinone reduction and oxidation signals which remained unchanged for several weeks.

Electroreduction of diazobenzene immobilized on electrodes modified with HYDPt-1. Diazobenzene is a small molecule with a photo- and electroactive azo group, which does not undergo adsorption on a bare glassy carbon electrode. However, when adsorbed on a HYDPt-1 modified electrode, diazobenzene remains stably attached to the surface, even after repeated transfers of the electrode into solutions of different pH and not containing the azocompound. Self-assembly of diazobenzene was carried out from a 1 mM methanol solution. Reduction of diazobenzene can be described as shown in the Scheme 2 :



20

Scheme 2

Figure 4 shows the cyclic voltammogram of diazobenzene adsorbed for 20 min on the HYDPt-1 - modified electrode, recorded in 0.1 M Tris / HClO_4 solution of pH 7.0. Curve 1 was recorded after adsorption of the diazobenzene for the same laps of time, but on the bare GCE. Curve 2 represents the electrode covered with hydrophobin, and curve 3 represents the electrode with both hydrophobin and diazobenzene. The well developed reduction and oxidation peaks do not change upon repeated cycling. The peak currents increase linearly with square root of the scan rate, indicating diffusion control rather than surface - immobilized species. Since the working solution does not contain diazobenzene, this dependence can be understood in terms of diffusion of diazobenzene

10

within the HYDPt-1 layer. Such behavior argues that, in the self-assembly process, the small and hydrophobic diazobenzene molecule penetrates into, and is immobilized in the HYDPt-1 layer.

5 The diazobenzene incorporated into the film is now being studied in our laboratories as a molecular switching device, based on the cis-trans isomerization taking place on UV irradiation.

10 Similar scan rate dependencies were observed for the Q0 molecule, which has the same headgroup as Q10 but does not possess an alkyl chain (Figure 5), and therefore can easily penetrate the HYDPt-1 layer. Curve 1 is a bare electrode in the presence of Q0, and curve 2 an electrode covered with hydrophobin and after adsorbtion of Q0.

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CLAIMS

1. Method of binding a compound to a sensor surface, said method comprising the step of adsorbing hydrophobin to said sensor surface, characterized in that the compound is chosen from the group consisting of i) an electroactive compound, an ii) a compound capable of being converted into an electroactive compound, said method comprising the steps of c) coating the electrode with a hydrophobin, and d) contacting the compound with the hydrophobin 10 to form a hydrophobin coating containing said compound in a non-covalently bound form.

2. Method according to claim 1, characterized in that the compound is a hydrophobic compound or a compound containing a hydrophobic anchor.

15 3. Method according to claim 1 or 2, characterized in that a second compound is bound covalently to the hydrophobin, the second compound being an electroactive compound.

4. Method according to any of the preceding claims, characterized in that a second compound is bound non-covalently to the hydrophobin, through a third compound being an intermediate compound having affinity for the second compound, said second compound being an electroactive compound.

20 5. Method according to claim 3 or 4, characterized in that the second compound is a redox enzyme.

25 6. Method according to claim 3 or 4, characterized in that the second compound is a light receptor.

7. Electrode coated with a hydrophobin, the hydrophobin containing a compound being an electroactive compound.

30 8. Electrode according claim 7, characterized in that the electrode is a glassy-carbon electrode, a glass electrode or a Thin Mercury Film Electrode.

9. Method of performing a measurement with an electrode coated with hydrophobin, characterized in that an electrode according to claim 7 or 8 is used.

35 10. Sensor comprising an electrode according to claim 7 or 8.

ABSTRACT

The present invention relates to a method of binding a compound to a sensor surface, said method comprising the step of adsorbing hydrophobin to said sensor surface. According to the invention, the compound is chosen from the group 5 consisting of i) an electroactive compound, an ii) a compound capable of being converted into an electroactive compound, said method comprising the steps of e) coating the electrode with a hydrophobin, and f) contacting the compound with the hydrophobin 10 to form a hydrophobin coating containing said compound in a non-covalently bound form.

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Fig 1a

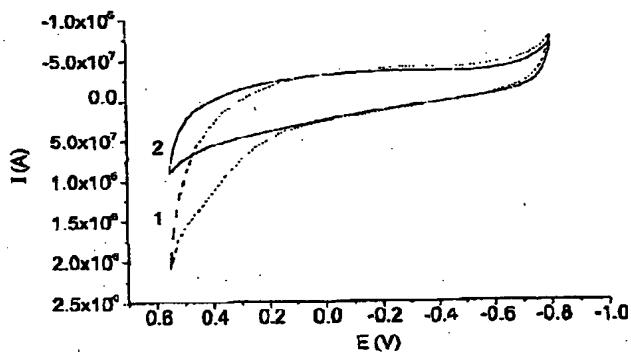


Fig 1b

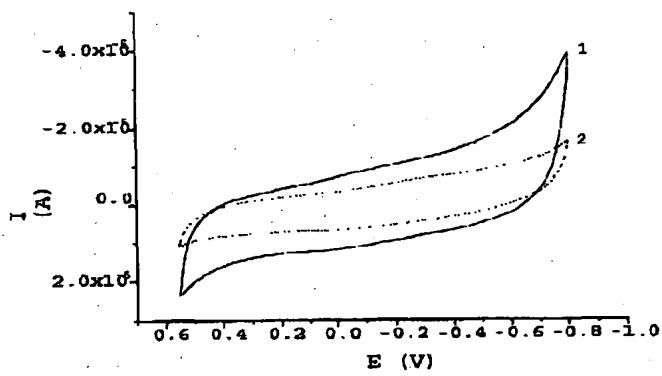
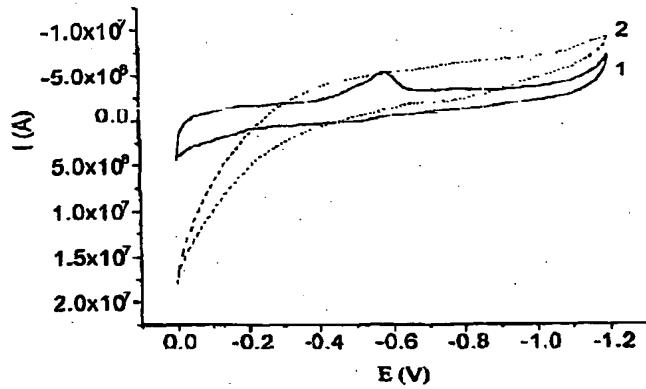


Fig 1c



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Fig 2a

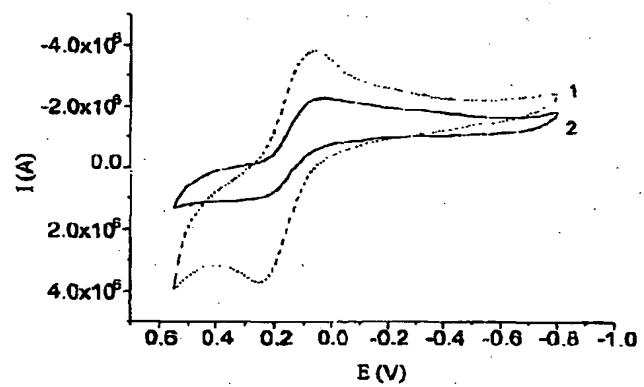
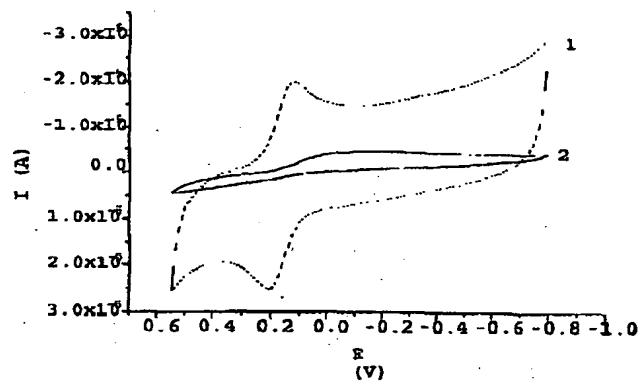


Fig2b



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Fig 3a

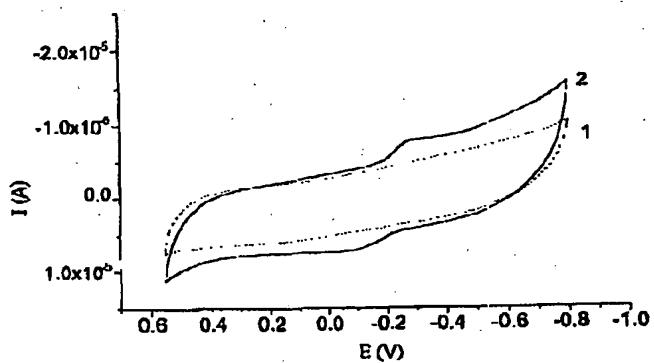
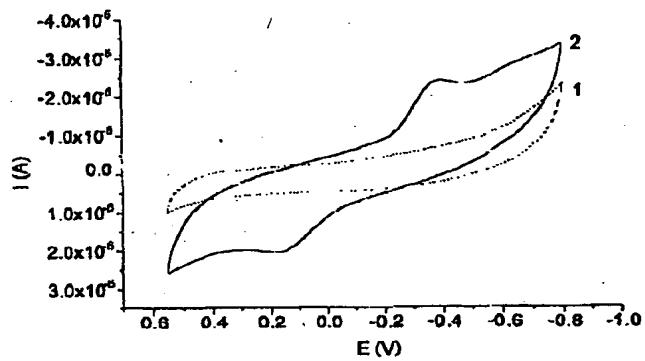


Fig 3b



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Fig 4

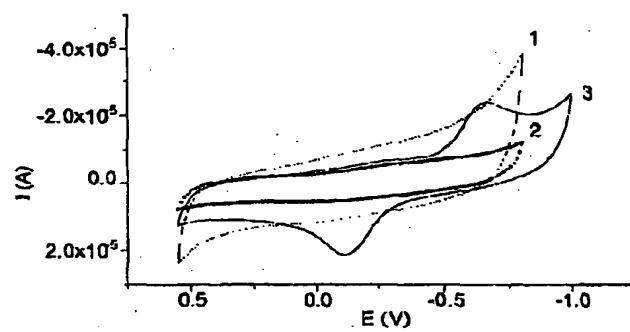
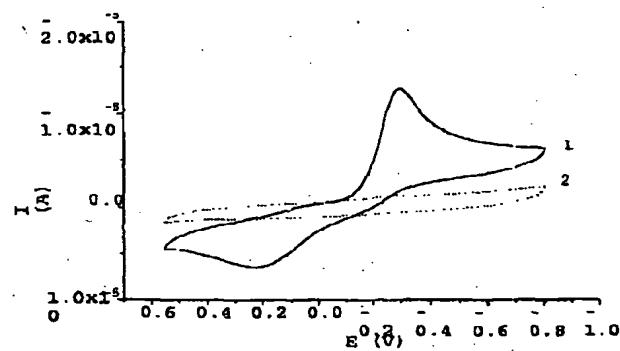


Fig 5



11

SEQUENCE LISTING

(Source: PCT/NL01/00268)

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10